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JNK3 MODULATORS AND METHODS OF USE

This application claims priority from provisional  
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Statement as to Federally Sponsored Research

This invention was made, in part, with support from  
the National Institutes of Health. The government may have  
certain rights in the invention.

10 Field of the Invention

The invention relates to screening assays for the  
detection of inhibitors of protein kinase expression or  
activity.

Background of the Invention

15 Apoptosis, or programmed cell death, is a prominent  
feature of the nervous system during normal development and  
in adult brain exposed to environmental stress (Kuida et  
al., Nature, 384:368-372, 1996; Ratan et al., Neurochem.,  
62:376-379, 1994; Raff et al., Science, 262:695-700, 1993).  
20 Stress-induced apoptosis has been implicated in a variety of  
neurological diseases (Thompson, Science, 267:1456-1462,  
1995) and requires *de novo* protein and RNA synthesis (Martin  
et al., J. Cell. Biol., 106:829-844, 1988; Oppenheim et al.,  
Dev. Biol., 138:104-113, 1990). Increased expression of c-  
25 Jun protein is associated with neuronal damage following  
global ischemia (Neumann-Haefelin et al., Cerebral Flow  
Metab., 14:206-216, 1994) or transection of nerve axons *in*  
*vivo* (Neumann-Haefelin, *supra*). Increased expression and  
phosphorylation of c-Jun have been observed *in vitro* prior  
30 to the apoptotic death of sympathetic neurons deprived of  
nerve growth factor (NGF) (Ham et al., Neuron, 14:927-939,  
1995). Moreover, expression of a dominant negative mutant

c-Jun, or treatment with c-Jun antibody protects NGF-deprived sympathetic neurons from apoptosis (Ham et al., *supra*; Estus et al., J. Cell. Biol., 127:1717-1727, 1994). However, the requirement of c-Jun for stress-induced neuronal apoptosis has not been tested *in vivo* since c-Jun deficient mice die during mid-gestation (Hilberg et al., Nature, 365:179-181, 1993).

Protein phosphorylation is one important mechanism involved in the activation of c-Jun in response to environmental stress signals (Whitmarsh et al., J. Mol. Med., 74:589-607, 1996). c-Jun N-terminal kinase (JNK, also known as SAPK) is a serine/threonine protein kinase that phosphorylates two residues (Ser-63 and Ser-73) on the NH<sub>2</sub>-terminal activation domain of c-Jun (Whitmarsh et al., *supra*; Dèrijard et al., Cell, 76:1025-1037, 1994; Kyriakis et al., Nature, 369:156-160, 1994). Map kinase kinase (MKK) 4 (also known as SEK1) is a direct activator of JNK in response to environmental stresses and mitogenic factors (Whitmarsh et al., *supra*; Dèrijard et al., *supra*; Nishina et al., Nature, 385:350-353, 1997; Yang et al., Proc. Nat. Acad. Sci. USA, 94:3004-3009, 1997; Sanchez et al., Nature, 372:794-798, 1994). JNK also phosphorylates ATF2 and other Jun-family proteins which function as components of the AP-1 transcription factor complex (Whitmarsh et al., *supra*; Gupta et al., Science, 267:389-393, 1995; Gupta et al., EMBO J., 15:2760-2770, 1996). The phosphorylation of these transcription factors by JNK leads to increased AP-1 transcriptional activity (Whitmarsh et al., *supra*). Conversely, the induction of AP-1 transcriptional activity is selectively blocked in cells lacking MKK4 (Yang et al., *supra*).

JNK has been implicated in the apoptosis of NGF-differentiated PC12 pheochromocytoma cells (Xia et al.,

Science, 270:1326-1311, 1995), one model system of neuronal cell death *in vivo* (Batistatou et al., J. Cell. Biol., 122:523-532, 1993). When differentiated PC12 cells are deprived of nerve growth factor (NGF), JNK activation is observed prior to apoptotic death (Xia et al., *supra*). Transfection studies using constitutively activated and dominant negative mutant components of the JNK signaling pathway established that JNK is involved in NGF withdrawal-induced apoptosis of PC12 cells (Xia et al., *supra*).

Ten JNK isoforms, resulting from alternative splicing of three different genes have been identified (Dèrijard et al., *supra*; Kyriakis et al., *supra*; Gupta et al., *supra*; Martin et al., Brain Res. Mol. Brain Res., 35:47-57, 1996). Although the JNK1 and JNK2 isoforms are widely expressed in murine tissues, including the brain, the JNK3 isoforms are predominantly expressed in the brain and, to a lesser extent, in the heart and testis.

#### Summary of the Invention

The invention is based on the discovery that mice lacking the JNK3 gene (JNK3(-/-)) develop normally and are resistant to excitotoxic damage, and that JNK3 plays a role in stress-induced seizure activity, AP-1 transcriptional activation, and kainate-induced apoptosis of hippocampal neurons. Thus, JNK3 is a mediator of kainate/glutamate excitotoxicity and a target for limiting or preventing excitotoxic damage.

The invention features a method of identifying a candidate compound that modulates JNK3 expression. The method includes the steps of incubating a cell that can express a JNK3 protein with a compound under conditions and for a time sufficient for the cell to express the JNK3 protein when the candidate compound is not present. The

expression of JNK3 is then measured in the cell in the presence of the compound. The expression of JNK3 is also measured in a control cell under the same conditions and for the same time. The amount of JNK3 expression in the cell  
5 incubated in the presence of the compound and in the control cell is compared. A difference in JNK3 expression indicates that the compound modulates JNK3 expression. In an embodiment of this method, the compound decreases JNK3 expression.

10 In another embodiment, the invention features a method of identifying a candidate compound that modulates JNK3 activity. The method includes the steps of incubating a cell that has JNK3 activity with a compound under conditions and for a time sufficient for the cell to express  
15 JNK3 activity when the candidate compound is not present. The activity of JNK3 is then measured in the cell in the presence of the compound. The activity of JNK3 is also measured in a control cell under the same conditions and for the same time. The amount of JNK3 activity in the cell  
20 incubated in the presence of the compound and in the control cell is compared. A difference in JNK3 activity indicates that the compound modulates JNK3 activity. In an embodiment of this method, the compound decreases JNK3 activity.

25 The invention also includes a method of identifying a compound that modulates the binding of a JNK3 polypeptide to a substrate. The method involves comparing the amount of a JNK3 polypeptide bound to a substrate in the presence and absence of a selected compound. A difference in the amount of binding of a JNK3 polypeptide to the substrate indicates  
30 that the selected compound modulates the binding of a JNK3 polypeptide. In an embodiment of this method, the binding of a JNK3 polypeptide to a substrate is decreased.

Another feature of the invention is a method for generating a totipotent mouse cell comprising at least one inactivated JNK3 gene. The method includes: a) providing a plurality of totipotent mouse cells; b) introducing into  
5 the cells a DNA construct that includes a mouse JNK3 gene disrupted by the insertion of a sequence into the gene, thus the disruption prevents expression of functional JNK3; c) incubating the cells so that homologous recombination occurs between the chromosomal sequence encoding JNK3 and the  
10 introduced DNA construct; and d) identifying a totipotent mouse cell that has at least one inactivated JNK gene.

Also featured in the invention is a method for generating a mouse homozygous for an inactivated JNK3 gene. The method includes the steps of: a) providing a totipotent  
15 mouse cell that contains at least one inactivated JNK3 gene; b) inserting the cell into a mouse embryo and implanting the embryo into a female mouse; c) permitting the embryo to develop into a neonatal mouse; d) permitting the neonatal mouse to reach sexual maturity; e) mating two of the  
20 sexually mature mice to obtain a mouse homozygous for the inactivated JNK3 gene. Such a mouse (homozygous JNK3(-/-)) is resistant to excitotoxic damage.

The invention also features methods of treating a patient having or at risk for a disorder of the nervous  
25 system involving excitotoxicity. The methods include administering to the patient a therapeutically effective amount of a compound that inhibits JNK3 expression, or a therapeutically effective amount of a compound that inhibits JNK3 activity. An antisense nucleic acid molecule or  
30 ribozyme can be used as the inhibitory compound. Disorders that can be treated by these methods include dementias including Alzheimer's disease, neurodegenerative diseases such as Huntington disease, cerebrovascular disorders such

as ischemia, amyotrophic lateral sclerosis, trauma including that caused by heat or cold, motor neuron disease, Parkinson's disease, or seizure disorders including epilepsy. Neuroendocrine disorders such as those that  
5 affect pituitary glands, adrenal glands, testis, or pancreas (e.g.,  $\beta$ -cells) can be treated with JNK3 modulators.

The invention also includes a transgenic non-human mammal having a transgene disrupting expression of a JNK3 gene, the transgene being chromosomally integrated into germ  
10 cells of the mammal. In an embodiment of the invention, the mammal is a mouse. The germ cells of the mammal can be homozygous for the transgene and the disruption of JNK3 gene expression can be the result of a null mutation. Another  
15 embodiment of the invention includes a cell line descended from a cell of the mammal having the transgene disrupting expression of a JNK3 gene.

A DNA construct comprising a disrupted mouse JNK3 gene is also featured in the invention. The disruption is by insertion of a sequence into the gene such that the  
20 disruption prevents or modifies the expression of functional JNK3.

Unless otherwise specified, "JNK3" can refer both to nucleic acids and polypeptides, such as the sequences shown in Figs. 1A-5B (SEQ ID NOS:1-12; see also, GenBank accession  
25 number: U34819 which corresponds to SEQ ID NO:1 and SEQ ID NO:2; U34820 which corresponds to SEQ ID NO:4 and SEQ ID NO:5; U07620 which corresponds to SEQ ID NO:7 and SEQ ID NO:8; L27128 which corresponds to SEQ ID NO:9 and SEQ ID NO:10; and L35236 which corresponds to SEQ ID NO:11 and SEQ  
30 ID NO:12). SEQ ID NO:3 and SEQ ID NO:6 represent deduced nucleotide sequences based on the presumed overlap between the sequences represented by SEQ ID NOS:1 and 4 with the sequence represented by SEQ ID NO:7. JNK3 also refers to

polypeptides that are at least 85% identical to the amino acid sequences listed above, and to the nucleic acids encoding those polypeptides. Examples of these sequences and methods of isolating them are found in Gupta et al.,  
5 *supra*, 1996; Kyriakis et al., *supra*; Martin et al., Brain Res. Mol. Brain Res., 35:45-57, 1996; and Mohit et al., Neuron, 14:67-78, 1995.

10 A "control" cell is a cell that is generally the same, e.g., genotypically and phenotypically, as the cell to which it is being compared (e.g., the cells can be sister cells), but which is not exposed to a test compound.

15 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described  
20 below. All publications, patent applications, patents, and other references mentioned herein are incorporated in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

25 Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

#### Brief Description of the Drawings

30 Fig. 1A is a schematic representation of the nucleic acid sequence of GenBank Accession No. U34819 (SEQ ID NO:1).

Fig. 1B is a schematic representation of the amino acid sequence of GenBank Accession No. U34819 (SEQ ID NO:2).



Fig. 1C is a schematic representation of the nucleic acid sequence of SEQ ID NO:3.

Fig. 2A-B is a schematic representation of the nucleic acid sequence of GenBank Accession No. U34820 (SEQ ID NO:4).

Fig. 2C is a schematic representation of the amino acid sequence of GenBank Accession No. U34820 (SEQ ID NO:5).

Fig. 2D is a schematic representation of the nucleic acid sequence of SEQ ID NO:6.

Fig. 3A-B is a schematic representation of the nucleic acid sequence of GenBank Accession No. U07620 (SEQ ID NO:7).

Fig. 3C is a schematic representation of the amino acid sequence of GenBank Accession No. U07620 (SEQ ID NO:8).

Fig. 4A is a schematic representation of the nucleic acid sequence of GenBank Accession No. L27128 (SEQ ID NO:9).

Fig. 4B is a schematic representation of the amino acid sequence of GenBank Accession No. L27128 (SEQ ID NO:10).

Fig. 5A is a schematic representation of the nucleic acid sequence of GenBank Accession No. L35236 (SEQ ID NO:11).

Fig. 5B is a schematic representation of the amino acid sequence of GenBank Accession No. L35236 (SEQ ID NO:12).

Fig. 6 is a diagram of the wild type JNK3 gene locus, targeting vector, and the mutated or disrupted JNK3 gene locus.

Fig. 7 is a bar graph showing the temporal responses of wild type and JNK3(-/-) mice to kainic acid (KA) injection.

Fig. 8 is a bar graph showing the temporal responses of wild type and JNK3(-/-) mice to pentetrazole (PTZ) injection.

Fig. 9A is a schematic representation of the nucleic acid sequence of murine c-Jun (GenBank Accession No. X12740; SEQ ID NO:13).

Fig. 9B is a schematic representation of the amino acid sequence of murine c-Jun (GenBank Accession No. X12740; SEQ ID NO:14).

Fig. 10A-B is a schematic representation of the nucleic acid sequence of murine c-Fos (GenBank Accession No. V00727; SEQ ID NO:15).

Fig. 10C is a schematic representation of the amino acid sequence of murine c-Fos (GenBank Accession No. V00727; SEQ ID NO:16)

Fig. 11 is a bar graph showing the level of KA-induced AP-1 activity at various times after KA induction as reflected by luciferase activity in JNK3(-/-) mice crossed with transgenic AP-1 luciferase mice.

Fig. 12 is a bar graph showing the level of KA-induced AP-1 activity as reflected by the relative level of luciferase activity in hippocampus (HP) and cerebellum (CB) of JNK3(-/-) mice and wild type(+/+) mice.

Fig. 13 is a diagram of the proposed chain of molecular events caused by KA leading to neuronal apoptosis.

Fig. 14 is a diagram of the trisynaptic connection within the hippocampal formation.

#### Detailed Description

JNK protein kinase phosphorylates c-Jun and subsequently increases AP-1 transcriptional activity in response to a specific group of stress signals (Whitmarsh et al, *supra*; Yang et al., *supra*). The neural-specific expression of JNK3 may render neurons particularly susceptible to physiological stress. In the experiments described herein, a remarkable resistance to kainic acid

(KA)-induced seizures and apoptosis has been observed in JNK3-deficient mice. The resistance to KA neurotoxicity may be due to the elimination of a specific stress-response pathway mediated by the JNK3 isoform of JNK protein kinase.

5 First, the administration of KA caused the phosphorylation of the NH<sub>2</sub>-terminal activation domain of c-Jun and markedly increased AP-1 transcriptional activity in wild-type, but not in JNK3-deficient mice. Second, there was prolonged expression of phosphorylated c-Jun within the most  
10 vulnerable area of the hippocampus, further indicating that JNK activity may lead to neuronal apoptosis.

The findings reported herein are consistent with the dependence of KA neurotoxicity on excitatory circuitry (Nadler et al., Brain Res. 195:47-56, 1980). Since JNK3 is  
15 widely expressed in the nervous system and its activity is increased by many different stress signals (Gupta et al., *supra*), JNK3 may be involved in stress-induced apoptosis caused by a wide range of environmental insults.

The identification of JNK3 as a critical mediator of  
20 KA-induced excitatory neurotoxicity has clinical implications. The amino acid sequence of mouse, rat and human JNK3 is highly conserved (Kyriakis et al., *supra*; Gupta et al., *supra*; Martin et al., *supra*; Mohit et al., Neuron. 14:67-78, 1995). Moreover, the expression of the  
25 human JNK3 gene is also restricted to the nervous system and neuroendocrine system, is widely expressed in many brain subregions (Gupta et al., *supra*; Mohit et al., *supra*). It is therefore likely that the human and rodent JNK3 protein kinases have related or identical physiological functions.  
30 Neurotoxicity of the excitatory amino acids has been implicated in many neurological disorders ranging from acute ischemia to chronic neurodegenerative diseases (Choi, Neuron, 1:623-634, 1988; Lipton et al., N. Engl. J. Med.

330:613-622, 1994; Rothman et al., Annu. Neurol. 19:105-111, 1986). Previous therapeutic strategies have been focused on the prevention of calcium influx through cell surface channels, such as the NMDA-type glutamate receptor. To  
5 date, these approaches have only met with mixed results (Lipton et al., *supra*). JNK3 is therefore a target for therapeutic interventions when excitatory neurotoxicity involves JNK3-mediated apoptosis.

In the experiments described *infra*, homologous  
10 recombination was used to generate JNK3-deficient mice, and their responses to noxious stimuli were examined. KA, a potent excitotoxic chemical, elicits limbic seizures and neuronal cell death. The neurotoxicity of KA derives from the direct stimulation of the glutamate receptor at  
15 postsynaptic sites, and an indirect increase in the release of excitatory amino acids from presynaptic sites. It is well-documented that systemic application of KA induces the expression of various cellular immediate early genes (cIEGs), including c-Jun and c-Fos. Thus, the application  
20 of KA triggers a stress-response pathway in the brain *in vivo*. The experiments detailed *infra* demonstrate that KA induces phosphorylation of c-Jun and an increase in AP-1 transcriptional activity in the brain of wild-type mice. However, these effects of KA are markedly suppressed in the  
25 brains of JNK3-deficient mice. Moreover, JNK3-deficient mice exhibit a remarkable resistance to KA-induced seizures and apoptosis of hippocampal neurons. These normal mice treated with KA represent a useful model of human disorders of the nervous system involving excitotoxicity.

30 Based on these experimental results, JNK3 was found to be an exceptional target for limiting excitotoxic damage. In particular, JNK3 is a target in screening protocols including protocols to screen for molecules that regulate

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JNK3 gene expression, JNK3 binding to its substrates, and JNK3 activity, as described below. The molecules found in these screens that effectively decrease JNK3 expression or activity are candidate drugs to be used to treat disorders of the nervous system involving excitotoxicity, including seizure disorders such as epilepsy, cerebrovascular disorders including ischemia, metabolic imbalance (e.g., hypoglycemia), injury due to extreme heat or cold, trauma (e.g., irradiation, spinal cord injury, pressure, and ionic imbalance), dementias such as Alzheimer's disease, Parkinson's disease, and neurodegenerative disorders (e.g., Huntington disease), and motoneuron disease (including amyotrophic lateral sclerosis) (Thompson, Science, 267:1456-1462, 1995; Coyle et al., Science, 262:689-695, 1993).

#### Methods of Screening for Molecules that Inhibit JNK3 Activity

The following assays and screens can be used to identify compounds that are effective inhibitors of JNK3 activity. The assays and screens can be done by physical selection of molecules from libraries, and computer comparisons of digital models of compounds in molecular libraries and a digital model of the JNK3 active site. The inhibitors identified in the assays and screens may act by, but are not limited to, binding to JNK3 (e.g., from mouse or human), binding to intracellular proteins that bind to JNK3, compounds that interfere with the interaction between JNK3 and its substrates, compounds that modulate the activity of a JNK3 gene, or compounds that modulate the expression of a JNK3 gene or a JNK3 protein.

Assays can also be used to identify molecules that bind to JNK3 regulatory sequences (e.g., promoter

sequences), thus modulating gene expression. See, e.g., Platt, *J. Biol. Chem.*, 269:28558-28562, 1994.

The compounds that can be screened by the methods described herein include, but are not limited to, peptides and other organic compounds (e.g., peptidomimetics) that bind to a JNK3 protein or inhibit its activity in any way. Such compounds may include, but are not limited to, peptides; for example, soluble peptides, including but not limited to members of random peptide libraries (see, e.g., Lam et al., *Nature* 354:82-84, 1991; Houghten et al., *Nature* 354:84-86, 1991), and combinatorial chemistry-derived molecular libraries made of D-and/or L- amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang et al., *Cell* 72:767-778, 1993), and small organic or inorganic molecules.

Compounds and molecules are screened to identify those that affect expression of a JNK3 gene or some other gene involved in regulating the expression of JNK3 (e.g., by interacting with the regulatory region or transcription factors of a gene). Compounds are also screened to identify those that affect the activity of such proteins (e.g., by inhibiting JNK3 activity) or the activity of a molecule involved in the regulation of JNK3.

Computer modeling or searching technologies are used to identify compounds, or identify modified compounds that modulate or are candidates to modulate the expression or activity of a JNK3 protein. For example, compounds likely to interact with the active site of the JNK3 protein are identified. The active site of JNK3 can be identified using methods known in the art including, for example, analysis of the amino acid sequence of a molecule, and from a study of complexes formed by JNK3 with a native ligand (e.g., ATF2 or

c-Jun). Chemical or X-ray crystallographic methods can be used to identify the active site of JNK3 by the location of a bound ligand such as c-Jun or ATF2.

The three-dimensional structure of the active site can be determined. This can be done using known methods, including X-ray crystallography, which can be used to determine a complete molecular structure. Solid or liquid phase NMR can be used to determine certain intra-molecular distances. Other methods of structural analysis can be used to determine partial or complete geometrical structures. Geometric structure can be determined with a JNK3 protein bound to a natural (e.g., c-Jun or ATF2) or artificial ligand which may provide a more accurate active site structure determination.

Computer-based numerical modeling can be used to complete an incomplete or insufficiently accurate structure. Modeling methods that can be used are, for example, parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups are necessary, and can be selected from force fields known in physical chemistry. Information on incomplete or less accurate structures determined as above can be incorporated as constraints on the structures computed by these modeling methods.

Having determined the structure of the active site of a JNK3 protein, either experimentally, by modeling, or by a combination of methods, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. The

compounds identified in such a search are those that have structures that match the active site structure, fit into the active site, or interact with groups defining the active site. The compounds identified by the search are potential  
5 JNK3 modulating compounds.

These methods may also be used to identify improved modulating compounds from an already known modulating compound or ligand. The structure of the known compound is modified and effects are determined using experimental and  
10 computer modeling methods as described herein. The altered structure is compared to the active site structure of a JNK3 protein to determine or predict how a particular modification to the ligand or modulating compound will affect its interaction with that protein. Systematic  
15 variations in composition, such as by varying side groups, can be evaluated to obtain modified modulating compounds or ligands of preferred specificity or activity.

Given the teachings herein, additional experimental and computer modeling methods useful to identify modulating  
20 compounds based on identification of the active sites of a JNK3 protein and related transduction and transcription factors can be developed by those skilled in the art.

Examples of molecular modeling systems are the QUANTA programs, e.g., CHARMM, MCSS/HOOK, and X-LIGAND,  
25 (Molecular Simulations, Inc., San Diego, CA). QUANTA provides a modeling environment for two dimensional and three dimensional modeling, simulation, and analysis of macromolecules and small organics. Specifically, CHARMM analyzes energy minimization and molecular dynamics  
30 functions. MCSS/HOOK characterizes the ability of an active site to bind a ligand using energetics calculated via CHARMM. X-LIGAND fits ligand molecules to electron density patterns of protein-ligand complexes. The program also



allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

Articles reviewing computer modeling of compounds interacting with specific proteins can provide additional guidance. For example, see, Rotivinen et al., *Acta Pharmaceutica Fennica* 97:159-166, 1988; Ripka, *New Scientist* 54-57 (June 16, 1988); McKinaly and Rossmann, *Ann. Rev. Pharmacol. Toxicol.* 29:111-122, 1989; Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp.189-193 (Alan R. Liss, Inc., 1989); Lewis and Dean, *Proc. R. Soc. Lond.* 236:125-140, 141-162, 1989; and, regarding a model receptor for nucleic acid components, see Askew et al., *Am. J. Chem. Soc.* 111:1082-1090. Computer programs designed to screen and depict chemicals are available from companies such as MSI (*supra*), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Gainesville, FL). These applications are largely designed for drugs specific to particular proteins; however, they may be adapted to the design of drugs specific to identified regions of DNA or RNA. Commercial sources of chemical libraries can be used as sources of candidate compounds. Such chemical libraries can be obtained from, for example, ArQule, Inc. (Medford, MA).

In addition to designing and generating compounds that alter binding, as described above, libraries of known compounds, including natural products, synthetic chemicals, and biologically active materials including peptides, can be screened for compounds that are inhibitors or activators.

Compounds identified by methods described above may be useful, for example, for elaborating the biological function of JNK3 gene products and in treatment of disorders in which JNK3 activity is deleterious. Assays for testing

the effectiveness of compounds such as those described herein are further described below.

In Vitro Screening Assays for Compounds that Bind to JNK3 Proteins and Genes

5           In vitro systems can be used to identify compounds that can interact (e.g., bind) to JNK3 proteins or genes encoding those proteins. Such compounds may be useful, for example, for modulating the activity of JNK3 polypeptides or nucleic acids, elaborating their biochemistry, or treating  
10 disorders caused or exacerbated by JNK3 expression. These compounds may themselves disrupt normal function or can be used in screens for compounds that disrupt normal function.

          Assays to identify compounds that bind to JNK3 proteins involve preparation of a reaction mixture of the  
15 protein and the test compound under conditions sufficient to allow the two components to interact and bind, thus forming a complex that can be detected and/or isolated.

          Screening assays for molecules that can bind to a JNK3 protein or nucleic acid can be performed using a number  
20 of methods. For example, a JNK3 protein, peptide, or fusion protein can be immobilized onto a solid phase, reacted with the test compound, and complexes detected by direct or indirect labeling of the test compound. Alternatively, the test compound can be immobilized, reacted with JNK3  
25 polypeptide, and any complexes detected. Microtiter plates can be used as the solid phase and the immobilized component anchored by covalent or noncovalent interactions. Non-covalent attachment may be achieved by coating the solid phase with a solution containing the molecule, and drying.  
30 Alternatively, an antibody specific for JNK3 is used to anchor the molecule to the solid surface. Such surfaces may be prepared in advance of use, and stored. JNK3 antibodies

can be produced using conventional methods such as those described in Coligan et al. (Current Protocols in Immunology, John Wiley & Sons, Inc., 1994, see Volume 1, chapter 2).

5           In the assay, the non-immobilized component is added to the coated surface containing the immobilized component under conditions that permit interaction and binding between the two components. The unreacted components are then removed (e.g., by washing) under conditions such that any  
10 complexes formed will remain immobilized on the solid phase. The detection of the complexes can be accomplished by a number of methods known to those in the art. For example, the nonimmobilized component of the assay may be prelabeled with a radioactive or enzymatic label and detected using  
15 appropriate means. If the non-immobilized entity was not prelabeled, an indirect method is used. For example, if the non-immobilized entity is a JNK3 polypeptide, an antibody against JNK3 is used to detect the bound molecule, and a secondary, labeled antibody is used to detect the entire  
20 complex.

          Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected (e.g., using an immobilized antibody specific for a JNK3 protein).

25           Cell-based assays can be used to identify compounds that interact with JNK3 proteins. Cell lines that naturally express such proteins or have been genetically engineered to express such proteins (e.g., by transfection or transduction with JNK3 DNA) can be used. For example, test compounds can  
30 be administered to cell cultures and the phosphorylation of ATF2 or c-Jun measured as described *infra*. A decrease in the amount of phosphorylation of a JNK3 substrate in the presence of the test compound compared to controls that do

not contain the test compound indicates that the test compound is an inhibitor of JNK3 activity.

Inhibitors of JNK3 expression that act on the JNK3 promoter can be identified using a chimeric gene in which genomic sequences including the JNK3 promoter are fused to a reporter, for example firefly luciferase. Cultured cells (including neurons) transformed with this DNA are screened for the expression of luciferase activity. Compounds that inhibit luciferase activity in this high throughput assay can be confirmed by direct measurement of the endogenous JNK3 protein (by Western blotting) and JNK3 mRNA (by Northern blotting) using methods known in the art (for example, see Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1994).

Candidate inhibitory compounds can be tested further in cell or tissue cultures as well as animal models. For example, cells expressing JNK3 are incubated with a test compound. Lysates are prepared from treated and untreated cells and Western blotted according to known methods. The blots are probed with antibodies specific for JNK3. A decrease in the amount of JNK3 expression in cultures treated with the test compound compared to untreated controls indicates that the test compound is a candidate for a drug to treat disorders associated with JNK3 expression.

#### Assays for Compounds that Interfere with JNK3/JNK3 Substrate Interactions

Molecules that disrupt the interaction between JNK3 and its substrates can be identified using assays that detect protein-protein interactions. For example, the yeast two-hybrid method detects protein interactions *in vivo*. However, an *in vitro* assay is preferable because candidate molecules may not be permeable to the yeast cell wall. An

example of an *in vitro* assay for such test molecules that  
disrupt the interaction between JNKC3 and a substrate  
includes the use of immobilized JNK3 or immobilized  
substrate (e.g., c-Jun) and incubation of the immobilized  
5 component with cell lysates or purified proteins in the  
presence and absence of a test molecule. In general, the  
test molecule is tested over a range of a 100 fold molar  
excess over the most abundant component (e.g., the component  
immobilized or in solution). If the test molecule is  
10 predicted to interact with the immobilized component of the  
assay, then it can be pre-incubated with that component  
before adding the cell lysate or purified protein. After  
washing away unbound material, the bound proteins are  
detected with antibodies (e.g., ELISA or Western blot) or  
15 through the use of labeled proteins (e.g. radioactive or  
fluorescent) using methods known in the art. Test molecules  
that decrease the amount of substrate bound to JNK3 are thus  
identified as molecules that interfere with JNK3/JNK3  
substrate interactions.

Assays for Compounds that Ameliorate the Effects of JNK3 in vivo

Compounds identified as above, or other candidate compounds that inhibit JNK3 activity *in vitro* may be useful for treating disorders involving JNK3 activity. These compounds can be tested in *in vivo* assays, for example, in animal models of disorders involving JNK3 activity. For example, transgenic mouse models of ALS have been described (Bruijn and Cleveland, *Neuropathol. Appl. Neurobiol.* 22:373-387, 1996; Dal Canto and Gurney, *Brain Res.* 676: 25-40, 1995; Cleveland et al., *Neurology* 47: Suppl 2, S54-61) as have transgenic models of Alzheimer's disease such as the PDAPP mouse and others (for examples, see Loring et al., *Neurobiol. Aging* 17:173-182, 1996). MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced dopaminergic neurotoxicity has been used as a model for Parkinson's disease in rodents and nonhuman primates (for example, Przedborski et al., *Proc. Nat'l. Acad. Sci. USA* 93:4565-4571, 1996).

Test compounds predicted to inhibit JNK3 activity are administered to animals, e.g., as described above, that serve as models for the various disease paradigms. Treated animals are then assayed for inhibition of JNK3 activity. Such assays may be indirect or inferential, for example, improved health or survival of the animal indicates the efficacy of a test compound. Assays can also be direct, for example, a decrease in JNK3 or c-Jun expression can be measured by Northern analysis of neural tissue removed from an animal treated with a test compound. A decrease in the amount of JNK3 mRNA present in the sample from treated animals compared to untreated controls indicates that the test compound is inhibiting JNK3 expression. A decrease in

the amount of c-Jun indicates that the test compound is inhibiting JNK3 expression or activity.

#### Antisense Constructs and Therapies

5 Treatment regimes based on an "antisense" approach  
involve the design of oligonucleotides (either DNA or RNA)  
that are complementary to JNK3 mRNAs. These  
oligonucleotides bind to the complementary mRNA transcripts  
and prevent translation. Absolute complementarity, although  
10 preferred, is not required. A sequence "complementary" to a  
portion of an RNA, as referred to herein, is a sequence  
sufficiently complementary to be able to hybridize with the  
RNA, forming a stable duplex; in the case of double-stranded  
antisense nucleic acids, a single strand of the duplex DNA  
may be tested, or triplex formation may be assayed. The  
15 ability to hybridize will depend on both the degree of  
complementarity and the length of the antisense nucleic  
acid. Generally, the longer the hybridizing nucleic acid,  
the more base mismatches with an RNA it may contain and  
still form a stable duplex (or triplex, as the case may be).  
20 One skilled in the art can ascertain a tolerable degree of  
mismatch by use of standard procedures to determine the  
melting point of the hybridized complex.

Oligonucleotides that are complementary to the  
5' end of the message, e.g., the 5' untranslated sequence,  
25 up to and including the AUG initiation codon, are generally  
most efficient for inhibiting translation. However,  
sequences complementary to the 3' untranslated sequences of  
mRNAs have also been shown to be effective for inhibiting  
translation (Wagner, Nature, 372:333, 1984). Thus,  
30 oligonucleotides complementary to either the 5' or 3' non-  
translated, non-coding regions of a JNK3 could be used in an  
antisense approach to inhibit translation of the endogenous

human homolog of JNK3 mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Examples of candidate antisense sequences for the 5' and 3' regions are;

- 5 5'-AAG AAA TGG AGG CTC ATA AAT ACC ACA GCT-3' (SEQ ID NO:17)  
and 5'-ATT GGA AGA AGA CCA AAG CAA GAG CAA CTA-3' (SEQ ID NO:18), respectively.

- 10 While antisense nucleotides complementary to the coding region of a JNK3 gene could be used, those complementary to transcribed untranslated regions are most preferred. Examples of this type of candidate sequence are 5'-TAA GTA AGT AGT GCT GTA TGA ATA CAG ACA-3' (SEQ ID NO:19) and 5'-TAC TGG CAA TAT ATT ACA GAT GGG TTT ATG-3' (SEQ ID NO:20).

- 15 Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation, but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3', or coding region of a JNK3 mRNA, antisense nucleic acids should be at least six  
20 nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, or at least 50 nucleotides in length.

- 25 Regardless of the choice of target sequence, *in vitro* studies are usually performed first to assess the ability of an antisense oligonucleotide to inhibit gene expression. In general, these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. In  
30 these studies levels of the target RNA or protein are usually compared with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared



with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide, and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA, or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule or hybridization. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (as described, e.g., in Letsinger et al., Proc. Natl. Acad. Sci. USA 86:6553, 1989; Lemaitre et al., Proc. Natl. Acad. Sci. USA 84:648, 1987; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, for example, PCT Publication No. WO 89/10134), or hybridization-triggered cleavage agents (see, for example, Krol et al., BioTechniques 6:958, 1988), or intercalating agents (see, for example, Zon, Pharm. Res. 5:539, 1988). To this end, the oligonucleotide can be conjugated to another molecule, e.g., a peptide, hybridization-triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethyl-

aminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, 5 N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 10 5-methyl-2-theouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 2-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide can also comprise at 15 least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

The antisense oligonucleotide can also include at 20 least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal, or an analog of any of these backbones.

25 The antisense oligonucleotide can include an  $\alpha$ -anomeric oligonucleotide. An  $\alpha$ -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gautier et al., Nucl. Acids. Res. 30 15:6625, 1987). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., Nucl. Acids Res. 15:6131, 1987), or a chimeric RNA-DNA analog (Inoue et al., FEBS Lett. 215:327, 1987).

Antisense oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (Nucl. Acids Res. 16:3209, 1988), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., Proc. Natl. Acad. Sci. USA 85:7448, 1988).

The antisense molecules should be delivered to cells that express JNK3 proteins *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

However, it is often difficult to achieve intracellular concentrations of the antisense molecule sufficient to suppress translation of endogenous mRNAs. Therefore, an approach may be used in which a recombinant DNA construct comprises an antisense oligonucleotide placed under the control of a strong *pol* III or *pol* II promoter. The use of such a construct to transfect target cells in a patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous JNK3 transcripts and thereby prevent translation of that mRNA. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally

integrated, as long as it can be transcribed to produce the desired antisense RNA.

Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Suitable promoters include, but are not limited to: the SV40 early promoter region (Bernoist et al., Nature 290:304, 1981); the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., Cell 22:787-797, 1988); the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. USA 78:1441, 1981); and the regulatory sequences of the metallothionein gene (Brinster et al., Nature 296:39, 1988). Constructs may also be contained on an artificial chromosome (e.g., mammalian artificial chromosome; MAC; Harrington et al., Nature Genet. 15:345-355, 1997).

The production of a JNK3 antisense nucleic acid molecule by any gene therapeutic approach described above results in a cellular level of JNK3 protein that is less than the amount present in an untreated individual.

## 25 Ribozymes

Ribozyme molecules designed to catalytically cleave JNK3 mRNAs can also be used to prevent translation of these mRNAs and expression of JNK3 mRNAs (see, e.g., PCT Publication WO 90/11364; Saraver et al., Science 247:1222, 1990). While various ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy specific mRNAs, the use of hammerhead ribozymes is

preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases:  
5 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art (Haseloff et al., Nature 334:585, 1988). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the  
10 5' end of the JNK3 mRNA, i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

Examples of potential ribozyme sites in human JNK3 include 5'-UG-3' sites which correspond to the initiator methionine codon at, for example, in human JNK3, about  
15 nucleotides 224-226, the codon for a downstream potential initiation site (nucleotides 338-340), and additional codons in the coding region, including nucleotides 698-670; 740-742; and 935-937.

The ribozymes of the present invention also include  
20 RNA endoribonucleases (hereinafter "Cech-type ribozymes"), such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS or L-19 IVS RNA), and which has been extensively described by Cech and his collaborators (Zaug et al., Science 224:574, 1984; Zaug et al., Science,  
25 231:470, 1986; Zug et al., Nature 324:429, 1986; PCT Application No. WO 88/04300; and Been et al., Cell 47:207, 1986). The Cech-type ribozymes have an eight base-pair sequence that hybridizes to a target RNA sequence, whereafter cleavage of the target RNA takes place. The  
30 invention encompasses those Cech-type ribozymes that target eight base-pair active site sequences present in JNK3 proteins.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, or targeting), and should be delivered to cells which express a JNK3 gene *in vivo*, e.g., the brain and spinal cord. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive *pol* III or *pol* II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous JNK3 mRNAs and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

For any of the above approaches, the therapeutic JNK3 antisense or ribozyme nucleic acid molecule construct is preferably applied directly to the target area (e.g., the focal site of activity in a seizure disorder, the hippocampus in Alzheimer's disease, the *substantia nigra* in patients with Parkinson's disease), but can also be applied to tissue in the vicinity of the target area or even to a blood vessel supplying the target area.

For gene therapy, antisense, or ribozyme JNK3 expression is directed by any suitable promoter (e.g., the human cytomegalovirus, simian virus 40, or metallothionein promoters), and its production is regulated by any desired mammalian regulatory element. For example, if desired, enhancers that direct preferential gene expression in cells under excitotoxic induction can be used to direct antisense JNK3 expression in a patient with a seizure disorder.

JNK3 antisense or ribozyme therapy is also accomplished by direct administration of the antisense JNK3 or ribozyme RNA to a target area. This mRNA can be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using an antisense JNK3

DNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of antisense JNK3 RNA to target cells is carried out by any of the methods for direct administration of therapeutic compounds described herein.

5 Methods of Treating Disorders Involving JNK3 Expression or Activity

10 The invention also encompasses the treatment of disorders, especially in mammals, such as humans, in which JNK3 plays a damaging role. A number of disorders of the nervous system involving excitotoxicity, such as seizure disorders (e.g., epilepsy), dementias such as neurodegenerative disorders (e.g., Alzheimer's disease, Huntington disease), cerebrovascular disorders such as ischemia, motor neuron disease (including ALS), injuries  
15 caused by extreme heat or cold, trauma (e.g., irradiation, spinal cord injury, pressure, and ionic imbalance), metabolic imbalance (e.g., hypoglycemia) and Parkinson's disease, can be treated by the methods described herein. Without limiting the invention by committing to any  
20 particular theory, a substantial number of neurologic disorders are attributable, at least in part, to excitotoxicity which is mediated by the JNK3 pathway. Thus, inhibitors of this pathway, identified as described above, are useful for treatment of disorders involving  
25 excitotoxicity.

Therapy can be designed to reduce the level of endogenous JNK3 gene expression, e.g., using antisense or ribozyme approaches to inhibit or prevent translation of a JNK3 mRNA; triple helix approaches to inhibit transcription  
30 of the gene; or targeted homologous recombination to inactivate or "knock out" a gene or its endogenous promoter. The antisense, ribozyme, or DNA constructs described herein

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can be administered directly to the site containing the target cells; e.g., specific regions of the brain or the spinal cord. Antibodies or fragments of antibodies that recognize JNK3 or a JNK3 substrate, and that have been  
5 modified to be expressed or otherwise enter the cell can also be used therapeutically.

#### Effective Dose

Toxicity and therapeutic efficacy of the compounds of the invention, e.g., compounds that modulate JNK3  
10 expression or activity, can be determined by standard pharmaceutical procedures, using either cells in culture or experimental animals to determine the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The  
15 dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Polypeptides or other compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be  
20 taken to design a delivery system that targets such compounds to the site of affected tissue to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and  
25 animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form  
30 employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially



from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the  $IC_{50}$  (that is, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

#### Formulations and Use

10           Pharmaceutical compositions for use in accordance with the present invention can be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

15           Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

20           For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (for example, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose);  
25           fillers (for example, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (for example, magnesium stearate, talc or silica); disintegrants (for example, potato starch or sodium starch glycolate); or wetting agents (for example, sodium lauryl sulphate). The  
30           tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or

they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (for example, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (for example, lecithin or acacia); non-aqueous vehicles (for example, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (for example, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

The preferred methods of administering the compositions of the invention are by direct delivery of the compounds to the central nervous system, preferentially to the brain, especially near to or directly at the site of the disorder, e.g., the hippocampus in the case of Alzheimer's disease, the substantia nigra in the case of Parkinson's disease, and the focal site for seizure disorders. Accordingly, administration may be into a ventricle, intrathecal, or intracerebral ventricular. For example, an Omayia reservoir-shunt with in-line filter can be surgically placed into the cisternal space. A therapeutic compound in an appropriate excipient (e.g., phosphate-buffered saline) is instilled into the shunt by injection on a prescribed basis.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds can be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, for example, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, for example, sterile pyrogen-free water, before use.

The compounds can also be formulated in rectal compositions such as suppositories or retention enemas, for example, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for

example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The therapeutic compositions of the invention can also contain a carrier or excipient, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. The nucleic acids, polypeptides, antibodies, or modulatory compounds of the invention can be administered by any standard route of administration. For example, administration can be parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, transmucosal, or oral. The modulatory compound can be formulated in various ways, according to the corresponding route of administration. For example, liquid solutions can be made for ingestion or injection; gels or powders can be made for ingestion, inhalation, or topical application. Methods for making such formulations are well known and can be found in, for example, "Remington's Pharmaceutical Sciences." It is expected that particularly useful routes



## Example 2. Targeted Disruption of the JNK3 Gene

To generate JNK3-deficient mice, a targeting vector was designed to replace an internal 4 kb MscI-SpeI JNK3 genomic fragment with a PGKneo cassette. A map of the JNK3 gene, the JNK3 targeting vector, and the predicted structure of the mutated JNK3 gene are shown diagrammatically in Fig. 6. Restriction enzyme sites are indicated (B, BamHI; Hp, HpaI; M, MscI; Nco, NcoI; R, EcoRI; Spe, SpeI). A 10-kb NotI-EcoRI (the NotI site was vector-derived) JNK3 fragment was cloned from a  $\lambda$  FixII phage library of a 129/Sv mouse strain (Stratagene Inc.). The targeting vector was constructed by inserting a 4.0 kb MscI fragment from the 5' end of the JNK3 genomic fragment, a 1.6 kb PGK-neo cassette (Negishi et al., Nature 376:435-438, 1995) and a 1.8-kb SpeI-NcoI fragment of the 3' end of the JNK3 fragment into pBluescript KS vector (Stratagene Inc.) using appropriate linkers. The targeting vector contains a 2.6-kb PGK tk cassette (Negishi et al., *supra*) flanking the 5' end of the JNK3 genomic sequence for negative-selection of mutant ES cells (Mansour et al., 336:348-352, 1988). The region replaced in the JNK3 gene by the targeting vector encompasses one and a half exons encoding amino acids 211 to 267 of JNK3 (as shown in Fig. 5B). This region includes the tripeptide dual phosphorylation motif Thr-Pro-Tyr (TPY) that is characteristic of the JNK group and required for protein kinase activity (Dèrijard et al., *supra*). The two hatched boxes shown in the JNK3 locus correspond to subdomains VIII and IX (encoding amino acid residues 189-267 in the JNK3 protein shown in Fig. 5B) of JNK3.

The targeting vector was linearized with NotI and electroporated into W9.5 embryonic stem (ES) cells. Genomic DNA from transfectants resistant to G418 (200 mg/ml) (Gibco BRL) and gancyclovir (2 mM) (Syntex, Palo Alto, CA) were

isolated and screened by Southern blot analysis. Southern blot analysis of 104 independent G418- and gancyclovir-resistant clones revealed three clones containing the desired homologous recombination event (targeting frequency 2.9%). Chimeric mice were generated by injecting these ES cells into C57BL/6 (B6) mouse blastocysts.

Southern blots of EcoRI-restricted DNA derived from the tails of these chimeric mice were probed with the radiolabeled 351 bp JNK3 probe. EcoRI digestion resulted in a 12 kb band corresponding to the wild-type (endogenous allele), and a 4.2 kb band corresponding to the mutant (disrupted allele).

Two clones mediated germline transmission of the disrupted JNK3 allele into the next generation of mice. Heterozygotes (+/-) were intercrossed to generate homozygous mutant mice (-/-) that were identified by Southern blot analysis of genomic DNA. Total RNA isolated from mouse brain was examined by Northern blot analysis. The blot was probed with a random-primed <sup>32</sup>P-labeled mouse JNK3 cDNA probe, then stripped and sequentially reprobed with mouse JNK1 and  $\beta$ -actin cDNA probes. The major JNK3 transcript in brain is 2.7 kb, and the JNK1 transcripts in mouse brain are 2.3 and 4.4 kb. Blots hybridized with a JNK3 cDNA probe detected transcripts in wild-type (+/+), but not in homozygous knockout (-/-) mice.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was used to confirm that JNK3 transcripts were absent in the homozygous JNK3 (-/-) brain. A JNK1 probe (447 bp) was amplified from mouse brain RNA by RT-PCR (Yang et al., *supra*) using the amplimers 5'-GTGTGCAGCTTATGATGCTATTCTTGAA-3' (SEQ ID NO:21) and

5' CGCGTCACCACATACGGAGTCATC-3' (SEQ ID NO:22). RT-PCR detection (Yang et al., *supra*) of JNK3 mRNA in mouse tissues was performed by RT-PCR using the amplimers:

5'-CTGGAGGAGTTCCAAGATGTCTACT-3' (SEQ ID NO:23) and

5 5'-TGGAAAGAGCTTGGGGAAGGTGAG-3' (SEQ ID NO:24) to yield a specific 537 bp DNA product. RNA isolated from mouse brain was amplified with primers specific for HPRT as a control. These experiments confirmed the absence of JNK3 transcripts in the homozygous JNK3 (-/-) brain.

10 Protein kinase assays were performed to show that JNK3 (-/-) mouse brain was deficient in JNK3 activity. In these experiments, JNK3 kinase activity in brain lysates was measured after immunodeletion of JNK1 and JNK2 by in-gel protein kinase assays using the substrate GST-cJun (Dèrijard  
15 et al., *supra*). When mouse hippocampal lysates (30 µg) from wild type (+/+) and homozygous knockout (-/-) brains were assayed, the 55 kD and 46 kD JNK3 isoforms were detected in wild type but not JNK3 (-/-) mice, confirming that JNK3 (-/-) mouse brain was deficient in JNK3 kinase activity.  
20 Together, these data demonstrate that the targeted disruption of the JNK3 gene resulted in a null allele.

The JNK3(-/-) mice were fertile and of normal size. Histological surveys of a variety of tissues revealed no apparent abnormality using hematoxylin and eosin (H & E)  
25 staining of heart, lung, thymus, spleen, lymph nodes, liver, kidney, and skeletal muscle. JNK3(-/-) and wild-type mouse brains were examined by immunocytochemical analysis of a pyramidal neuronal marker (MAP-2), interneuronal markers calbindin and parvalbumin, an astrocyte marker (glial  
30 fibrillary acidic protein; GFAP; Hsu et al., J. Histochem. Cytochem. 29:577-580, 1981), and Nissl's stain (Hsu et al., *supra*). These studies revealed that JNK3(-/-) mice had apparently normal development and structural organization of



the brain. A comparable number of motor neurons were found in the facial nucleus in wild-type and JNK3(-/-) mice (2150-2300 neurons per nucleus at postnatal day 10, n=4). The neurons were identified by morphology and were counted by a double-blind assay of serial sections throughout the facial nuclei of wild-type and JNK3(-/-) mice. Thus, there is no apparent developmental abnormality, including cell death, in JNK3 (-/-) mice.

Example 3. JNK3 Deficient Mice are Resistant to KA-Induced Seizures

JNK3(-/-) mice and their wild-type littermates were injected intraperitoneally (i.p.) with 30 mg/kg KA to induce seizures (Ben-Ari, supra). In wild-type mice, the administration of KA first induced "staring spells" with abnormal body posture, then progressed to head nodding ("wet-dog shakes"), fore-paw tremor, rearing, loss of postural control, and eventually, continuous convulsions. The seizure activities typically subsided one hour after injection. Wild-type and heterozygous mice developed motor symptoms of seizures, including rearing, at 30 to 40 minutes post-injection. The JNK3 (-/-) mice, in contrast, developed much milder symptoms, mainly consisting of "staring" spells and occasional myoclonic tremors. At this dose, JNK3 (-/-) mice did not develop grand mal seizures and recovered much faster than did wild-type and heterozygous mice. JNK3(-/-) mice developed seizures of comparable severity to wild-type mice only at higher dosages of KA (45 mg/kg, i.p.). However, at this high dose of KA, more than 60% of wild-type mice died from continuous tonic clonic convulsions, while all of the JNK3(-/-) mice survived. These results indicate that JNK3(-/-) mice were resistant to the effect of the excitotoxin KA. Further, JNK3(-/-) mice recovered from the

drug administration more rapidly than wild-type mice (Fig. 7). Seizure classifications as shown in Figs. 7 and 8 are: 1, arrest of motion; 2, myoclonic jerks of the head and neck, with brief twitching movements; 3, unilateral clonic activity; 4, bilateral forelimb tonic and clonic activity; and 5, generalized tonic-clonic activity with loss of postural tone, often resulting in death.

Example 4. Resistance to Pentetrazole (PTZ)-Induced Seizures

Since the resistance to KA-induced seizures varied between littermates (+/+ and +/- are less resistant than -/- mice), the observed differential susceptibility cannot be attributed to a difference between mouse strains (Schauwecker and Steward, Proc. Nat. Acad. Sci. USA 94:4103-4108, 1997). However, the resistance of JNK3 deficient mice to KA-induced seizures could be due to decreased drug penetration across the blood-brain barrier or an increased GABA (gamma-aminobutyric acid) inhibitory postsynaptic potential (IPSP), or the ablation of a specific signal transduction pathway mediated by the JNK3 protein kinase. To distinguish between these possibilities, the response of JNK3 (-/-) and wild-type mice to another epileptogenic agent, pentetrazole (PTZ) (Sigma), was examined. PTZ was selected due to its ability to induce seizures by blocking the GABA-IPSPs (Ben-Ari et al., Neurosci. 6:1361-1391, 1981).

JNK3 (-/-) mice and wild-type littermates developed seizures of comparable severity at all tested dosages of PTZ (30, 40, 50, 60 mg/kg, i.p.; Fig. 8). Moreover, unlike the slow progression of motor symptoms seen in the KA-induced seizures, PTZ induced abrupt general tonic-clonic seizures within five minutes after injection, presumably reflecting

that its epileptogenic mechanism works solely through extracellularly inhibition of the GABA-IPSP. Thus, the differential susceptibility to KA toxicity in JNK3(-/-) mice can neither be explained as a consequence of poor drug  
5 delivery to the nervous system nor by potent GABA-IPSPs in the neural circuit. Furthermore, we examined the expression of the kainate-type glutamate receptor subunits GluR5-7 (Pharmingen cat. no. 60006E) by immunocytochemistry using standard methods.

10           Pyramidal neurons in the hippocampal CA1 subfield were most prominently labeled by the Glu5-7 antibody. Both wild-type and JNK3(-/-) mice showed prominently labeled apical dendrites arising from lightly labeled somata in the CA1 subfield of the hippocampus, a pattern similar to the  
15 primate hippocampus (Good et al., Brain Research 624:347-353, 1993). In addition to kainate-type subunit GluR5-7, the expression pattern of the GluR1 subunit that is essential to various glutamate receptor subtypes and the intracellular calcium-binding proteins parvalbumin and  
20 calbindin that may buffer the influx of extracellular calcium were also indistinguishable between JNK3(-/-) and wild-type mice. Together, these results indicate no apparent structural abnormality that might be responsible for the resistance of JNK3(-/-) mice to KA-induced  
25 excitotoxicity.

#### Example 5. Attenuation of KA-Induced Phosphorylation of c-Jun

The systemic administration of KA in wild-type mice may induce a stress-response pathway mediated by the JNK3  
30 protein kinase. To explore this possibility, the expression of the immediate-early genes *c-fos* and *c-jun* (Morgan et al., Annu. Rev. Neurosci. 14:421-451, 1991; Smeyne et al., Nature

363:166-169, 1993; Kasof et al., J. Neurosci. 15:4238-4249, 1995) was examined to determine whether KA imposed an equivalent level of noxious stimulation on wild-type and JNK3(-/-) mice. Total RNA was extracted from the hippocampi of mice sacrificed before and at 0.5, 2, 4, or 8 hours after KA injection (30 mg/kg, i.p.), and Northern blots were probed with murine *c-fos* and *c-jun* probes. The *c-Jun* probe was a 207bp fragment corresponding to nucleotides 888-1094 (Fig. 9) of the murine *c-Jun* cDNA. The *c-Fos* probe was a 347 bp fragment of the murine *c-Fos* gene (exon 4; base pairs 2593-2939) (Fig. 10). Both JNK3(-/-) and wild type mice exhibited a comparable level of rapid induction of *c-fos* and *c-jun* transcripts, which gradually declined four hours after injection.

To further define this phenomenon, the distribution of KA-induced *c-Fos* and *c-Jun* immunoreactivity along the synaptic circuit of the hippocampus was examined. In these experiments, homozygous mutant and control wild-type mice were killed and fixed by transcardial perfusion of 4% paraformaldehyde at 2 or 6 hours after the injection of KA (30 mg/kg, i.p.). Brains from both groups were removed, post-fixed for one hour, and sectioned on a Vibratome (40 mm thick). Tissue sections were processed by immunocytochemistry to detect the expression of *c-Jun* (Santa Cruz, cat# sc-45), *c-Fos* (Santa Cruz, cat# sc-52), and phospho-specific *c-Jun* (Ser-73) (New England Biolabs, #9164S). Sections were floated in a solution of the primary antibody (diluted 200X in PBS) and incubated overnight at room temperature. Secondary antibody incubation, avidin-biotin conjugated peroxidase (Vectastain Elite ABC kit, Vector Lab.), and DAB (3, 3'-diaminobenzidine, Sigma) reactions were performed using standard procedures (Hsu et al., *supra*). In the absence of KA, there was no detectable

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c-Fos expression and only a few c-Jun-positive cells within  
the dentate gyrus. Two hours after KA injection (30 mg/kg,  
i.p.), there was a large increase in c-Fos immunoreactivity  
throughout the hippocampal region that was the same in both  
5 wild-type and JNK3(-/-) mice. Simultaneously, there was an  
increase in the number of c-Jun-positive cells in the  
dentate gyrus and the CA3 region of the hippocampus in both  
wild type and JNK3(-/-) mice. By six hours after KA  
injection, the expression of c-Jun extended to the CA1  
10 region in both wild-type and JNK3(-/-) mice. The induction  
of c-Fos and C-Jun is generally accepted as an indicator of  
neuronal activity following noxious stimulation (Morgan et  
al., *supra*). The comparable induction level, time-course,  
and distribution of c-Jun and c-Fos-labeled cells suggests  
15 that JNK3(-/-) and wild type mice were subject to an  
equivalent level of noxious stress by systemic  
administration of KA.

C-Jun is activated by phosphorylation of the NH<sub>2</sub>-  
terminal activation domain by JNK. The expression of  
20 phosphorylated c-Jun provides another measure of whether  
JNK-like activity was present in JNK3(-/-) mice. The  
expression of phosphorylated c-Jun was examined using an  
antibody raised against c-Jun phosphorylated at Ser-73, one  
of the sites phosphorylated by JNK (Whitmarsh et al., *supra*;  
25 Dèrijard et al., *supra*; Kyriakis et al., *supra*). Prior to  
challenge with KA, no cells were labeled by the antibody in  
either wild type or JNK3(-/-) mice. By two hours after KA  
injection, there was a high level of phosphorylated c-Jun in  
the dentate gyrus and the CA3/CA4 region of the hippocampus  
30 in wild-type mice. In contrast, only a trace amount of  
phosphorylated c-Jun was detected in the JNK3(-/-) mice.  
Thus, there was either a decreased level or less sustained  
phosphorylation of c-Jun in the JNK3(-/-) mice.

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In addition, there was a dynamic change of the distribution of phosphorylated c-Jun in the wild type mouse hippocampus. By six hours after KA injection, the expression of phosphorylated c-Jun subsided in the dentate gyrus and progressed to a restricted area in the hippocampal CA3 region. Under higher magnification, it was apparent that the expression of phosphorylated c-Jun surrounded a focus of cell destruction. In contrast, no labeling of phosphorylated c-Jun was detected in the JNK3(-/-) mice at the same time point. The hippocampal CA3 region is well documented as the most vulnerable structure to the KA excitotoxicity, presumably due to both a high KA binding affinity (Berger et al., *supra*) and a potent excitatory synaptic connection between CA3 pyramidal neurons (Westbrook et al., Brain Research 273:97-109, 1983). These results indicate that JNK3 is required for the phosphorylation of c-Jun induced by KA.

Example 6. Attenuation of KA-Induced AP-1 Transcriptional Activity

20 Since the phosphorylation of c-Jun is an important initial event during the induction of AP-1 transcriptional activity (Whitemarsh et al., *supra*; Yang et al., *supra*), whether the observed attenuation of c-Jun phosphorylation would lead to decreased induction of AP-1 transcriptional activity in JNK3 (-/-) mice was examined. JNK(-1-) mice were crossed with transgenic AP-1 luciferase (AP1-luc) mice (Rincon et al., Embo. J. 13:4370-4381, 1994) and progeny back crossed. The JNK3(-/-)/API-Luc(-/+) mice were used in experiments with JNK3(+/+) mice to compare the level of KA-induced AP-1 transcriptional activity in the presence or absence of JNK3. The AP1-luc mice contain the firefly luciferase gene under the control of four copies of a

consensus AP-1 binding site in the context of the minimal rat prolactin promoter. It has been established that the expression of luciferase in these mice is due to the presence of the AP-1 regulatory element.

5 In the luciferase assay, mice were sacrificed at intervals after injection of KA (30 mg/kg, i.p.), and relative luciferase activity compared with that detected in hippocampal lysates obtained from mice injected with vehicle (saline). Mice were decapitated, brains were dissected, and  
10 brain tissues were immediately lysed in buffer containing 25mM Hepes pH 7.4, 1% TRITON®X-100, 1mM EDTA, 1mM phenylmethyl sulfonyl fluoride, and 10 µg/ml leupeptin (Promega, Madison, WI). Luciferase activity was measured as described in Rincon and Flavell (Embo. J. 13:4370-4381,  
15 1994). The injection of KA (30 mg/kg, i.p.) caused a large induction of AP-1 transcriptional activity in the hippocampus of wild-type mice, as evidenced by the induction of luciferase activity. Luciferase activity in wild type mice was detectable by six hours, gradually increased to the  
20 peak at three days, and persisted for at least seven days (Fig. 11). Control experiments demonstrated that the injection of vehicle (saline) did not cause induction of luciferase activity in the AP1-luc mice.

The relative luciferase activity in the hippocampus  
25 and cerebellum prepared from wild-type (+/+) and JNK3 (-/-) mice was measured following KA injection. The results are shown in Fig. 12. Each time point represents the mean of three to five (+ SEM) individual animals. The induction of luciferase activity was most prominent in the hippocampus,  
30 where a markedly greater induction of phosphorylation of c-Jun was observed, as compared to the cerebellum and the cerebral cortex. The induction of AP-1 activity was significantly reduced in the JNK3 (-/-) mice with the

AP1-luc transgene as compared to the wild type mice. At 15 hours after injection of KA, there was approximately four-fold greater AP-1 activity in the hippocampus of wild-type mice compared with JNK3(-/-) mice. At three days after injection, the AP-1 activity was more than six times higher in the hippocampus of wild-type compared with JNK3(-/-) mice. Together, these data demonstrate that the disruption of the JNK3 gene suppressed KA-induced phosphorylation of c-Jun and AP-1 transcription activity in the hippocampus *in vivo*.

#### Example 7. Resistance to KA-induced Apoptosis

One unique feature of KA among other epileptogenic agents is its potency in inducing neuronal cell death (Ben-Ari, *supra*; Schwob et al., *supra*). Since this property of cell destruction is paralleled by a sustained level of AP-1 transcriptional activity, it has been suggested that AP-1 mediates KA -induced-neuronal death (Kasof et al., *supra*; Schwarzschild et al., J. of Neurosci. 17:3455-3466, 1997). Wild-type and JNK3(-/-) mouse brains were therefore examined after treatment with KA to determine whether the attenuation of AP-1 transcriptional activity in JNK3 (-/-) mice altered the extent of neuronal damage (Ben-Ari, *supra*; Ben-Ari et al., *supra*; Schwob et al., Neurosci. 5:991-1014, 1980).

These experiments were performed as follows. Wild-type and JNK3 (-/-) mice were killed and fixed by transcardial perfusion of 4% paraformaldehyde and 1.5% glutaraldehyde three days after the injection of KA (30 mg/kg, i.p.). Semithin and thin sections of brain were prepared using a Vibratome and embedded in Epon. Tissue blocks were prepared using a microtome with a diamond tube for 1  $\mu$ m-thick semithin sections examined by toluidine blue staining, and for ultrathin sections examined by electron



microscopy. Nissl's stain was used for initial examination of damage to the hippocampus (Kluver et al., J. Neuropath. Exp. Neuro. 12:400-403, 1953). GFAP immunocytochemistry was also used to assess cell destruction in the hippocampus (Hsu et al., supra). Nissl's staining was also performed as described above. TUNEL assays, used to evaluate apoptosis, were performed using cryostat sections (50 $\mu$ m) of cerebral hemispheres that were cryoprotected with sucrose. The TUNEL assay was modified from the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay (Gavrieli et al., J. Cell. Biol. 119:493-501, 1992). Briefly, tissue sections, directly mounted on a salinated slide, were permeablized with 2% TRITON<sup>®</sup> X-100 (20 minutes at room temperature) and then incubated for nick end-labeling for 2 hours at 37°C using 0.32 U/ $\mu$ l TdT (Boehringer Mannheim, cat# 220582) and 2 $\mu$ M digoxigenin-11-dUTP (Boehringer Mannheim, cat# 1573152) in a final volume of 40  $\mu$ l. The tissues were incubated with anti-digoxigenin antibody (Boehringer Mannheim, cat# 1333062) diluted 500-fold, and processed for immunocytochemistry using standard procedures (Hsu et al., supra).

The damage to the hippocampus caused by KA was initially examined by Nissl's stain. The KA-induced cell loss caused either a breach of staining of the pyramidal neurons in the CA3 region or a diffuse sparse staining throughout the CA1 subfield. To corroborate the cell destruction revealed by Nissl's stain, the TUNEL method was applied to detect apoptosis. Groups of small pyknotic nuclei and positively TUNEL-labeled cells were found in the hippocampal CA3 subfield devoid of Nissl's staining. Similarly, a high percentage of pyramidal neurons showing pyknotic nuclei and shattered apical dendrites and numerous strongly TUNEL-labeled cells were located in the hippocampal

CA1 subfield which exhibited decreased Nissl's staining. Since the TUNEL method and the pyknosis morphology only indicated the extent of cell damage at one time point of a dynamic process, immunostaining of damage-induced GFAP was also used as an independent assessment of the extent of cell destruction in the hippocampus. Consistent with the patterns of Nissl's, toluidine, and TUNEL staining, an increased number of strongly GFAP-labeled astrocytes was found either in the hippocampal CA3 or CA1 regions. Thus, a combination of Nissl's stain, GFAP immunocytochemistry, TUNEL method, and toluidine stain of semithin sections was used to classify the KA-induced damage in the mouse hippocampus.

A total of 17 wild-type and 18 JNK3(-/-) mice were examined. Results are shown in Table 1 (below). The table was compiled from two sets of data. First, wild-type (n=11) and JNK3 (-/-) (n=10) mice were sacrificed on the fifth day after a single injection of KA (30 mg/kg, i.p.). Second, wild-type (n=6) and JNK3 (-/-) (n=8) mice received an injection of KA (30 mg/kg, i. p.) for five consecutive days and examined two days following the final injection. The severity of the hippocampal damage in wild-type mice was comparable in experiments using both protocols. The ratio of no cell loss/CA3 lesion/CA3+CA1 lesion was 2/7/2 in the single-injection experiments, and 2/2/2 in the multiple injection experiments.

TABLE 1 Kainate-induced neuronal damage (number of animals)

	JNK3 genotype	+/+	-/-
5	No perceivable cell loss	4	18
	Selective CA3 cell loss	9	0
	Including CA1 cell loss	4	0

10 The hippocampal CA3 region was the most susceptible to KA-induced damage in the wild-type mice (9/17; 53%). Cell loss was indicated by decreased crystal violet staining in the CA3 region. Using the TUNEL method (Gavrieli et al., *supra*), which identifies DNA fragmentation in the dying

15 cells, groups of labeled cells were found in the damaged region. A cluster of pyknotic nuclei was found in the CA3 region in toluidine-stained semithin sections. As a result of KA-induced damage, there was selective glial proliferation confined to the CA3 region, as indicated by

20 the strong immunostaining of GFAP. In some wild-type animals, massive cell loss was observed throughout the entire hippocampal CA1 region (4/17; 24%). Similarly, damage to the CA1 region was revealed by decreased crystal violet staining, positively TUNEL-labeled cells, pyknotic

25 nuclei, shattered apical dendrites of pyramidal neurons, and both hypertrophy and proliferation of GFAP-positive astrocytes.

In contrast, there was no apparent hippocampal damage in any of the JNK3(-/-) mice examined (n=18). The

30 pattern of the Nissl's stain, TUNEL assay, toluidine blue staining of semithin sections, and GFAP immunostaining of the hippocampal region in the JNK3(-/-) mice was indistinguishable from that of untreated wild-type mice.

Moreover, although JNK2(-/-) mice developed seizures of comparable severity at the sublethal dose of 45 mg/kg KA (a dose that is lethal for more than 60% of wild-type mice due to continuous convulsions), cell damage was nevertheless  
5 found in a much smaller percentage of animals (2/15, 13%;  $p < 0.005$  by chi-square analysis, d.f.=1).

Methods of assessing apoptosis (e.g., TUNEL assay) can be used to evaluate whether JNK3 modulator is affecting apoptosis.

10 Example 8. Electron Microscopic Analysis of Ultrastructural Changes Associated with KA-Induced Neuronal Damage

Cortical neurons *in vitro* undergo either apoptosis or necrosis depending on the extracellular concentration of the glutamate analog N-methyl-D-aspartate (NMDA) (Bonfoco et  
15 al., Proc. Natl. Acad. Sci. USA 92:7162-7166, 1995). The distinction between apoptosis versus necrosis in KA-induced neuronal damage is critical since necrosis is generally thought to represent a consequence of acute mechanical insult that is incompatible with an active cell death  
20 program involving *de novo* protein synthesis. The TUNEL results (*supra*) indicate the involvement of apoptosis. To further examine whether the neuronal death *in vivo* due to KA induction was apoptotic or necrotic, electron microscopy was employed to investigate the ultrastructural changes in the  
25 degenerated hippocampal neurons. The microscopic analysis suggested a series of morphological changes indicating neuronal damage in the wild-type mouse as a consequence of apoptosis. The initial event after KA injection (30 mg/kg i.p.) appeared to be compaction and segregation of chromatin  
30 in pyramidal neurons into electron-dense masses that abutted on the inner surface of the nuclear envelope. In contrast, the nuclei of the hippocampal neurons in the JNK3(-/-) mice

following KA injection contained homogeneous electron-lucent euchromatin. At later stages, in wild type mice, there was convolution of the nuclear outline and condensation of the cytoplasm. The double-layered structure of the nuclear envelope in wild type mice remained largely intact in all of these morphological stages. Eventually the degenerated neurons disintegrated resulting in numerous membrane-bounded apoptotic bodies. These morphological features are all consistent with the hallmarks of apoptosis (Kerr et al., Br. J. Cancer 26:239-257, 1972). Thus, it appeared that KA triggered a genetic program within the damaged neuron leading to apoptosis, which was abrogated in JNK3-deficient neurons.

These results suggest that KA-induced phosphorylation of the NH<sub>2</sub>-terminal activation domain of c-Jun leads to increased AP-1 transcriptional activity and neuronal apoptosis. Without limitation to a particular theory, a proposed chain of molecular events caused by KA that lead to neuronal apoptosis is shown in Fig. 13.

Although systemic administration of KA causes cell damage predominantly localized in the hippocampal CA3 area, the significance of JNK3 in stress-induced neuronal apoptosis is not only restricted to this region. Several lines of evidence indicate that the particular vulnerability of the CA3 hippocampal neurons to KA is due to their unique cellular and synaptic properties. First, the hippocampal CA3 and CA4 regions have the highest density of KA-receptors (Berger et al., Neurosci. Lett. 39:237-242, 1983). Second, the recurrent synaptic excitation is particularly potent in the hippocampal CA3 region (Miles et al., J. Physiol. (London) 373:397-418, 1986). The recurrent excitation of the CA3 pyramidal neurons may sustain JNK3 signaling and therefore rapidly induce KA excitotoxicity. The observed

progression of c-Jun phosphorylation from the dentate gyrus to the CA3 region is reminiscent of the synaptic circuitry of the hippocampus. A diagram of the trisynaptic connection within the hippocampal formation is shown in Fig. 14. The first synaptic relay (1) is from the afferent perforant path (pp) onto the granule cell of the dentate gyrus (DG). The second relay (2) follows the mossy fiber (mf) from the dentate gyrus to the CA3 hippocampal neurons. The third relay (3) is from the hippocampal CA3 to the CA1 region along the Schaffer collaterals (Sch). There are recurrent synaptic interactions of pyramidal neurons in the CA3 region.

Example 9. Assays for Detection of Inhibitors of JNK3 Protein Kinase Activity

Inhibitors of JNK3 can be identified in protein kinase assays. These assays can be performed using JNK3 purified from tissue (e.g., brain) or with recombinant enzyme. The recombinant JNK3 can be isolated from bacteria, yeast, insect, or mammalian cells using standard procedures. Assays of endogenous (natural) JNK3 are known in the art and assays of recombinant JNK3 have been described previously (Gupta et al., EMBO J. 15:2760-2770, 1996).

The protein kinase activity of JNK3 can be measured using ATP and protein substrates for JNK3 in an *in vitro* assay. These substrates include, but are not limited to, the transcription factors ATF2 and Elk-1 (Gupta et al., 1996, *supra*). The incorporation of phosphate into the substrate can be measured by several methods. One example is to measure the incorporation of radioactive phosphate (e.g.,  $^{32}\text{P}$ ) into the substrate. The incorporation into the substrate can be measured following removal of unincorporated radioactivity by precipitation with

trichloroacetic acid and recovery on phosphocellulose paper or by polyacrylamide gel electrophoresis. The radioactivity can be monitored by scintillation counting, phosphorimager analysis, or by autoradiography. In general, methods for automated high throughput screens would not use radioactive materials. For this purpose a method is used to detect the phosphorylated substrate without a radioactive probe. In one approach the electrophoretic mobility of the substrate is examined. For example, ATF2 demonstrates a marked reduction in electrophoretic mobility following phosphorylation by JNK on Thr-69 and Thr-71 (Gupta et al., Science 267:389-393, 1995).

A second approach is to detect the phosphorylation of the substrate using immunochemical methods (e.g. ELISA). Antibodies that bind specifically to the phosphorylated substrates are prepared (monoclonal and polyclonal) and are commercially available (e.g., New England Biolabs, Promega Corp., and Upstate Biotechnology Inc.). The extent of substrate phosphorylation is then measured by standard ELISA assay using secondary antibodies coupled molecules suitable for to spectrophotometric or fluorometric detection using methods known in the art.

Molecules that inhibit JNK3 can be identified in a high throughput screen. A molecule that is a preferred candidate to treat excitotoxic disorders inhibits JNK3, but not other protein kinases, including related MAP kinases. Candidate molecules once identified can be optimized using combinatorial chemical methods or by the synthesis of related molecules. These molecules represent candidate drugs that can be tested for JNK3 therapy.

Example 10. Assays for Detection of Inhibitors of JNK3  
Activation

5 The JNK protein kinases are activated by dual  
phosphorylation on Thr and Tyr within protein kinase sub-  
domain VIII (Davis, Trends Biochem. Sci. 19:470-473, 1994).  
These sites of activating phosphorylation are conserved in  
JNK3 (Gupta et al., 1996, *supra*). Molecules that inhibit  
the activation of JNK3 by interfering with the  
phosphorylation of JNK3 can be identified by measurement of  
10 JNK3 activation in the presence and absence of candidate  
molecules. Cells expressing JNK3, e.g., neuronal cells,  
neuroendocrine cells, or cells that are engineered to  
express recombinant JNK3 (Gupta et al., 1996 *supra*), are  
exposed to environmental stress (e.g., depolarization,  
15 excitotoxic agents, UV radiation, heat, and anoxia) to  
activate JNK3. The state of JNK3 activation can be assessed  
by several methods. For example, JNK3 can be isolated,  
washed free of the candidate inhibitor, and the activation  
state of JNK3 monitored by protein kinase assay (*supra*).  
20 Alternatively, the activation of JNK3 can be probed using  
immunological methods using antibodies that bind to the Thr  
and Tyr phosphorylated (activated) form of JNK3. Antibodies  
that bind specifically to the Thr and Tyr phosphorylated  
enzyme can be prepared (monoclonal and polyclonal) and are  
25 commercially available (e.g., from New England Biolabs and  
Promega Corp.). The extent of substrate phosphorylation can  
then be measured by a standard ELISA assay using secondary  
antibodies coupled to spectrophotometric or fluorometric  
detection.



### Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate  
5 and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

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